



# Evaluation of miRNA 34a-3p Genes Expression Level Associated with Breast Cancer in Females

<sup>1</sup>Huda M. Taher, <sup>2</sup>Ismail H. Aziz

<sup>1</sup>Fallujah Teaching Hospital for Women and Children/Al-Anbar Health Directorate, Fallujah

<sup>2</sup>Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

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**Abstract:** MicroRNAs (miRNAs) are short non-coding RNA (approximately 22 nucleotides) that plays an important role in gene regulation. The study aims to evaluate the expression of serum (miR-34a-3p) in the Iraqi population as more efficient biomarkers. Circulating serum miRNA-34a-3p expression was measured using RT-qPCR in 50 patients of breast cancer compared to 50 healthy controls. The results showed the expression of miRNA-34a-3p non-significant ( $p=0.38$ ) in patients as compared to control. According to histopathological type 96 % were Invasive ductal carcinoma (IDC), 4 % Invasive Lobular Carcinoma (ILC) of all breast cancer patients. Estrogen positive receptors (ER) were in (74%) (37/50) of the cases and progesterone positive receptors (PR) in (72%) (36/50) of the cases. Also out 20 of 50 cases (20%) positive and (60%) (30/50) for her-2/neu expression. It was concluded that Luminal A molecular subtype is the most common type in Iraqi female patients with breast cancer.

**Key words:** Breast cancer, MicroRNA-34a-3p, gene expression, molecular subtype.

**Corresponding author:** (E-mail: [huda.taher2100m@ige.uobaghdad.edu.iq](mailto:huda.taher2100m@ige.uobaghdad.edu.iq)).

## Introduction

Cancer is widely known as a silent killer and continues to list as one of the leading causes of death worldwide, claiming millions of lives every year (1). Breast cancer (BC) is the most prevalent cancer in female in both developing and developed countries (2).

BC is a heterogeneous disease with a broad spectrum of pathological, clinical plus molecular features. As stated by molecular features, breast cancers are sectioned to various subtypes, according to activation of hormone receptors (estrogen receptor and progesterone receptor), human epidermal growth factors receptor 2 (HER2), and/or BC mutations (3).

Numerous prognostic factors for breast cancer have been well documented (4,5).

Therefore, Breast cancer is generally classified by different histopathological and clinical criteria for different destinations, including prognosis (6).

Since breast cancer is one of the most fatal malignancies in females worldwide, various factors play a role in the improvement and promotion of the malignancy, major of them involve changes in the expression of specific genes, several evidences suggesting that (microRNAs) can regulate expression of specific genes performance as new technique for predicting and identifying BC patients, microRNAs which can regulate and control signaling pathways negatively or positively and affecting tumor genesis and other aspects of cancer progression, particularly breast cancer(7). MicroRNA (miRNA) is single-stranded RNAs with a length of

18-25 nucleotides. Accumulative evidences from numerous biological experiments demonstrates that miRNAs play an essential and significant role in many biological processes such as regulation of gene expression by post-transcriptionally binding to 5'untranslated regions (UTR), coding sequences, or 3'UTR of target messenger RNAs (mRNAs) (8).

Additionally, it is assessed that in mammals, about one-third of all protein coding genes' events are controlled *via* miRNAs (9). The miR-34 family members, *miR-34a* is more prevalent than *miR-34b/c* in cancer research. Nevertheless, the detailed downstream and upstream mechanisms of *miR-34a* remain ambiguous (10).

Among the miRNAs, *miR-34a* is a pivotal anti-oncogene miRNAs, Wnt1 is a target of *miR-34a*. High expression of miR-34a restrains the proliferation of cancer stem cells, EMT and promotion of breast tumor via the inactivation of the pathway(7). MiRNAs that could upregulate tumor-suppressing pathways remain under expressed such as *miR-7*, *miR-34a*(11).

#### Materials and methods

The study enrolled from November 2022 to January 2023 in the laboratories of the Baghdad University's genetic engineering and biotechnology institute. The samples were collected from patients who were diagnosed with breast cancer and consulted Al-Andalus Specialist Oncology Hospital in Baghdad and Al-Anbar Specialized Center for Cancer treatment.

Blood samples were collected from 50 Iraqi female patients with pathologically confirmed breast cancer and the healthy control samples were provided by (n=50) healthy females. Then venous blood was taken from patients and healthy groups five milliliters (mL); all patients diagnosed with BC were placed in gel tubes for 30 minutes at room temperature, and then centrifuged for 10 minutes to obtain serum for molecular assay.

#### Protocol of microRNA extraction

Total RNA, including microRNA, was extracted from the sample according to the protocol of TRIzol™ Reagent, 0.2 mL of chloroform add to the aqueous phase containing RNA, 0.5 mL of Isopropanol was added for RNA precipitated as white gel-like Pellet, 0.5mL of 70% ethanol was added for RNA washing. Finally, Pellet was rehydrated in 50µl of Nuclease Free Water and then incubated in a water bath at 55–60°C for 10–15 minutes.

#### Reverse Transcription for complementary DNA (c DNA) synthesis

RNA sample 4 µl was mixed with 1 µl stem-loop RT primers of miR-34a-3p. The Primers were designed in this study by Using the NCBI Gene Bank database and miRBASE, RT-qPCR primers were synthesized by Macrogene (South Korea), primers set adopted in the current study listed in the (Table 1).

**Table (1): Primer Sequence for *miRNA34a-3p* and *miRNA-16* Gene Expression.**

Primer Name	Sequence
<i>miR-34a-3p</i> RT-primer	5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACAGGGCA -3'
<i>miR-34a-3p</i>	F 5'- GGTTTTTTTTCAATCAGCAAGTATAC -3'
Universal Reverse	R 5'- GTGCAGGGTCCGAGGT -3'
<i>miR-16-1</i> RT-primer	5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACCGCCAAT-3'
<i>miR-16-1</i>	F 5'- GGTTTTTTTTTAGCAGCACGTAAT -3'

### Quantitative real-time polymerase chain reaction (RT-q PCR)

RT-qPCR was performed using SYBR Green PCR Kit (Synthol /Russia) following the manufacturer's instructions. The quantitative reaction was performed as 10  $\mu$ L total reaction volume, containing 5  $\mu$ L of SYBR Green Master Mix, 0.5  $\mu$ L of specific miRNA primer, forward and reverse, 2  $\mu$ L of RNase-free water and 2  $\mu$ L of cDNA, and performed using a Real-Time PCR system (Synthol /Russia).

The cycling conditions were set as follow: 95 °C for 1 min (1 cycle), 45 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20s. MiRNA expression levels were presented in terms of fold change normalized by the house keeping gene *miR-16-1* using the formula  $2^{-\Delta\Delta CT(12)}$ .

### Statistical analysis

Analysis of data was carried out using International Business Machine Statistical package for the Social Sciences (IBM SPSS– version 28). The results were analyzed statistically, and the values were expressed as Mean + S.E.T-test test was used to significant compare between two samples.

Chi-square test was used to significant compare between percentages. Statistical significance was considered whenever the P value was equal or less than 0.05.

### Results and discussion

The data show a higher significance ( $P \leq 0.0001$ ) between invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC),

Ductal 48 (96%) cases and 2 (4%) cases were lobular. Moreover, Many sex hormones including estrogen, progesterone and others including have been accused to take part in breast cancer development. The hormonal results of 50 BC patient presented that ER receptors were positive in 74% (37/50) of the cases and PR positive receptors in 72% (36/50) of the cases, while It was statistically significant positive association with getting BC ( $P=0.001$ ) and ( $P=0.002$ ) respectively.

On the other hands, HER-2 receptors were positive in 40% (20/50) of the cases, however statistically these relations did not reach the level of significance.

This study involved 100 females, who were enrolled in the study; consist of 50 females with BC of diagnosis during chemotherapy, along with 50 healthy females to serve as a control group. All BC samples were divided into five groups according to age, these groups ranged from  $\leq 30$  year old to upper than 60 year.

The age distribution of females involved in control group showed a mean of about  $35.8 \pm 11.7$  years. The mean of female's age with BC was  $49.6 \pm 10.3$  years, that significant increase in grouped (41-50) ( $p=0.01$ ) and significant in grouped upper than 60 ( $p=0.03$ ) as compared to control groups. Among these 50 Breast cancer females 28 (46%) were aged less than 50 years, and 22 (44 %) were upper than 50 year as shown in (Table 2).

Table (2): Distribution of sample study according to Age groups.

Age group	Patients	Control	p-value
$\leq 30$	2	21	0.0001
31-40	9	14	0.29
41-50	17	5	0.01
51-60	15	9	0.22
>60	7	1	0.03
p-value	0.005	0.0001	

According to the result, it was found that 28 (56 %) of total patients had a positive family history, while the

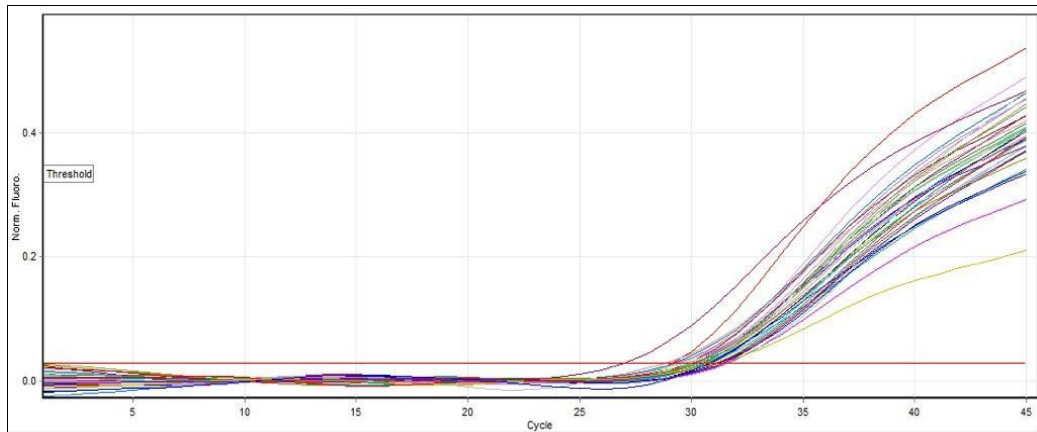
remaining 22 (44 %) did not, females with BC was non-significant as compared to control group (Table 3).

**Table (3): Distribution of sample study according to family history**

Cases	Positive No.(%)	Negative No.(%)	X2	P-value
Patients	28(56%)	22(44%)		0.39
Control	6(12%)	44(88%)	28.8	0.0001
<b>(P≤0.01) Highly Significant, NS: Non-Significant</b>				

Besides, the molecular experiment of *miR-34a-3p* expression was performed to detect the amplification plots of *miR-34a-3p* and

*miR-16* (reference gene) to find the threshold cycle (Ct) value for each. The curves of *miR-34a-3p* have shown in (Figure 1).



**Figure (1): *miR-34a-3p* dissociation curves by q PCR Samples included all study groups.**

Statistical analysis exposed non-significant in the mean of relative expression in the serum *miR-34a-3p* in breast cancer patients ( $p=0.38$ ) and

Calculating gene expression was done depending on Pfaffi ratio formula (12). As shown in (Table 4).

**Table (4): Comparison between serum *miR-34a-3p* expression between control and breast cancer groups.**

Cases	Mean of folding	P-value
Patients	1.91+- 0.39	0.38
Control	1.04+- 0.09	
<b>NS: Non-Significant</b>		

This study results showed that breast cancer risk happens at any age but it was increase in the middle age of female's life, and that also confirms the need for screening and early detection of breast cancer. According to recent Iraqi study done by Ibrahim in 2022, mentioned that The highest percentage of breast cancer cases was found in the fourth age group which reached 46% of

total patients, followed by 26% for the fifth age group and 22% for the third age group, while the lowest percentage was observed in the youngest age group which was 4% with a highly significant difference(13) ( $P\leq 0.01$ ). Likewise, the molecular subtypes result was similar to study shown that Luminal-A accounts higher percentage and more than 50% of invasive BC (14).

Although, Abbas (2022) found that (70%) of females with BC aged more than 50 years, The mean of females age with BC was high significantly increased as compared to control groups (15) . Besides, Abed *et al.* (2020) noted most of the patients were (40-49) years with a percentage of 42.85% and the lowest were with age less than 20 as the percentage was 2.8 % (16).

Correspondingly, The age developed give the cells more time to turn faulty or mutate to grow into cancer or older age have been exposed to more sunlight, cigarette smoke, chemicals and other cancer causing agents for a long Time . Iraq has the greatest risk of breast cancer among all countries, which may be due to the sociopolitical circumstances of the country (chemical warfare, bombings, etc (17).

As said by the family history, This study results disagreement with the other Iraqi studies for instance such as Al-saqabi and his collgues (2022) who found that 19 (38 %) of total patients had a positive family history, while the remaining 31 (62 %) did not, Abbas (2022) noted that the frequency of a family history only 11cases (7.33%)(13,15).

Hence, Having a family history of cancer increases the probability of developing the condition compared to people who do not have a family history; The strength of this association depends on how closely related the family members are The delay in cancer diagnosis in Iraq was influenced by a number of contributing variables. Iraq's healthcare system has been affected by the war, economic sanctions, and political issues. Because of the damage imposed to government healthcare organizations, the disruption of supply chains, and the "brain drain" of important medical personnel, this

reduced the quality of healthcare services (18).

A recent Iraqi study by Hussain and Lafta (2021) found the biological-chemical warfare in the following years (1980–88, 1990–91,2003–2006 and 2014–2016) left significant quantities of uranium and radiation throughout the area, has unique effects, including environmental contamination and a rise in the incidence of BC (19).The family history was studied because development of breast cancer is influenced by a number of inherited factors, *BRCA1 - BRCA2* dominant autosomal mutations (20,21).

The results of the present study showed that microRNA was successfully extracted from the serum samples of the patient and control groups. Statistical analysis revealed non- significant in *miRNA-34a-3p* expression.

This results are closely near to a novel study done by Mansoori *et al* in (21) establish that *miR-34a-3p* levels are reduced in breast tumors compared to adjacent normal tissues and that this additively predicts poor patient survival (22). And the primary results of meta-analysis study on the expression of *miR-34a-3p* and BC risk done by Imani *et al* in 2017 established there were no significant associations between *miR-34a-3p* levels and BC susceptibility for all genetic models (23).

This results is clearly agree with several studies, done by Si *et al* in 2016 establish that *miR-34a-3p* expression is reduced in BC, and they suggesting that aberrant expression of this miRNA could impact BC progression (24). Besides, Adams and his colleagues suggested that downregulation of this miRNA in several types of malignancies, including BC, suggests that *miR-34a-3p* actions as a tumor suppressor miRNA(25). Li *et al.*

demonstrated that of *miR-34a-3p* is downregulated in BC tumor (26).

Importantly, *MiR-34a-3p* is down regulated in most human cancers such as breast, colon, kidney, ovary, prostate, and skin cancers(27). However, only some tumor suppressor (TS) miRNAs that are often downregulated in cancer cells induce a robust phenotype. This has been demonstrated in various miRNA families that have received substantial attention, including the known tumoral suppressor's *let-7*, *miR-15/16*, *miR-34a-3p*, and *miR-200*. The tumor suppressive role of these miRNAs is demonstrated by the repression of their target oncogenic mRNA network, leading to an inhibition of tumor genesis (18).

### Conclusion

Our observations from this study found that the expression level of *miR-34a-3p* in breast cancer patients showed that not significant in comparison with healthy controls, therefore, it is not correlated with breast cancer. Also, the results of this study showed that the incidence of Ductal breast cancer is higher significance than Lobular subtype Iraqi BC patients.

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